

POST-TRANSCRIPTIONAL GENE SILENCING BY DOUBLE- STRANDED RNA

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Imagine being able to knock out your favourite gene with only a day's work. Not just in one model system, but in virtually any organism: plants, flies, mice or cultured cells. This sort of experimental dream might one day become reality as we learn to harness the power of RNA interference, the process by which double-stranded RNA induces the silencing of homologous endogenous genes. How this phenomenon works is slowly becoming clear, and might help us to develop an effortless tool to probe gene function in cells and animals.

One of the most effective ways to determine the biological function of a protein is to examine the phenotype of organisms that contain mutations in its encoding gene. Typically, such mutations are either created in a directed manner, using homologous recombination, for example in yeast or mammals, or identified by searching collections of randomly mutagenized organisms for lesions in a gene of interest, as is commonly done in worms and flies. Alternative methods for silencing specific genes have also provided potentially powerful approaches. Antisense methods, using either DNA or RNA, are relatively straightforward techniques for probing gene function; however, these methodologies have consistently suffered from the spectre of artefacts that arise from questionable specificity and incomplete efficacy. Recently, a new method for silencing specific genes has emerged¹. In diverse organisms, double-stranded (ds)RNAs have been shown to inhibit gene expression in a sequence-specific manner. This biological process, termed RNA interference, or RNAi, shows several features that border on the mystical. In *Caenorhabditis elegans*, gene silencing by RNAi can be initiated simply by soaking worms in dsRNA or by feeding worms *Escherichia coli* that express the dsRNA². RNAi is a potent method, requiring only a few molecules of dsRNA per cell to silence expression. Not only can silencing spread from the digestive tract of worms to the remainder of the organism, but the effect can also be

transmitted through the germ line for several generations³. These discoveries have intrigued the biological community — an interest that is also fuelled by the increasing power of RNAi as a tool to convert the accumulating hordes of genomic information into a deeper understanding of biological function. In this review, we highlight some of the recent progress that has been made towards understanding the mechanisms that underlie dsRNA-induced gene silencing and we briefly discuss ways in which this biological phenomenon is being harnessed as an experimental tool.

The discovery of RNAi

The discovery of RNAi grew out of a desire to use antisense approaches to probe gene function in *C. elegans*. In an effort to determine the function of the *par-1* gene, Guo and Kemphues injected antisense *par-1* RNA into worms⁴. This created the expected phenotype — embryonic lethality. However, a serious paradox was raised by the observation that injection of the control, sense-orientation RNA created an identical phenotype. With hindsight, this was reminiscent of observations that had been made in plants, in which increased gene dosage also caused loss of gene expression. However, at the time (six years ago), the inhibition of gene expression was thought to be caused by the saturation of the factors that are needed for *par-1* translation.

The key breakthrough came when Fire and Mello⁵,

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RNASE1

A ubiquitous vertebrate RNase that is activated indirectly by double-stranded RNA. This enzyme lacks the sequence specificity of RNA interference.

NON-CELL-AUTONOMOUS
A mutation that behaves non-cell-autonomously is one that affects cells other than those that possess the mutation.

PENETRANCE

The proportion of genetically mutant organisms that show the mutant phenotype. If all genetically mutant individuals show the mutant phenotype, then the genotype is said to be completely penetrant.

EPINETRIC

Transmission of phenotypes by mechanisms other than changes in DNA sequence.

NUCLEAR RUN-ON ASSAY

Direct measurement of transcriptional activity of a gene by incorporation of labelled UTP into its mRNA.

S-AZADENOXYCYTIDINE

A potent and specific inhibitor of DNA methylation.

who had also observed that either sense- or antisense-oriented RNAs inhibited gene function, asked whether injection of both the sense and the antisense strands into worms might give an additive, and so more complete, effect. Shockingly, the mixture of sense and antisense strands silenced expression of a target gene roughly tenfold more efficiently than either strand alone. Interpreting this dsRNA-induced effect as a new phenomenon, the authors named the process RNA interference, or RNAi.

The ability of dsRNA to affect gene expression was already well known in mammals; here, a series of interferon-inducible pathways respond to dsRNA by inhibiting translation through the action of a dsRNA-activated protein kinase (DAK or PKR)¹. The key difference between this response and RNAi was their respective specificity: the PKR response inhibited gene expression globally, whereas RNAi had a specific effect on gene expression. A more specific dsRNA response in mammalian cells had also been proposed to operate through the localized activation of a ribonuclease (RNASE1) that responds indirectly to dsRNA². The latter pathway might have provided a plausible explanation for RNAi, as it was clear that the targeted mRNA was destroyed in response to dsRNA. However, a series of startling observations made it clear that RNAi was mechanistically distinct from any previously known dsRNA response. One of the first indications that RNAi was a novel biological phenomenon was the potency of its effect. Injection into the worm of only a few molecules of dsRNA per cell was sufficient to almost completely silence the expression of a specific gene. Furthermore, the effect seemed to be non-cell-autonomous or, at the very least, systemic³. Injection of dsRNA into the gut of the worm caused silencing throughout the animal, and also in the F₁ progeny⁴. Transmission could occur through either the male or female germ line with almost complete penetrance.

The heritable nature of RNAi was compatible with the idea that dsRNA exerted its effect through the epigenetic suppression of chromosomal loci. However, several lines of evidence indicated that dsRNA might have induced gene silencing at the post-transcriptional rather than at the transcriptional level. For example, only dsRNAs corresponding to transcribed regions of genes could induce silencing^{5,6} — even dsRNAs directed against intronic regions were largely ineffective⁷.

Although the odd phenomenology of RNAi was not compatible with any previously known biological process, the authors of the original studies made some mechanistic predictions that, to their credit, have survived recent genetic and biochemical studies. They proposed that the key to the interference process is the recognition of the target mRNA by the dsRNA or a derivative thereof. Because the incoming dsRNA need not be present at stoichiometric levels, they proposed the existence of either a highly efficient catalytic machine or an amplification step. In the former case, they postulated that the machinery of RNAi would search out homologous mRNAs, which would subsequently be destroyed by a nuclease that was directed to them by base-pairing interactions. In the latter case, they pro-

posed an amplification of the signal, possibly through a replicative expansion of either the dsRNA or its derivative. The nature of the derivative that was either to direct the nuclease or to function as the template for amplification remained a mystery, because it could not be detected in worms that were undergoing silencing. As discussed below, a candidate for such an entity has been identified, first in plants and subsequently in *Drosophila melanogaster*. Ironically, a corresponding molecule has only recently been found in *C. elegans*⁸.

RNAi and cosuppression

While the use of RNAi as a reverse-genetic tool took the worm world by storm, continuing studies in plants revealed that previously studied silencing phenomena were reminiscent of RNAi in worms. In retrospect, it is clear that the phenomenon of RNAi was first discovered in plants (not in worms) as post-transcriptional gene silencing, or PTGS^{9,10} (BOX 1).

With the development of tools for introducing transgenes into plants, attempts were made to engineer plants with more desirable characteristics. Among such efforts were those of Rich Jorgensen and his group, who tried to deepen the purple colour of petunias^{11,12}. Unexpectedly, many plants failed to express the introduced transgenes. Furthermore, if the transgene contained homology to a cellular gene, the endogenous copy was also often silenced. The term ‘cosuppression’ was coined to describe the ability of exogenous elements to also alter the expression of endogenous genes.

There seemed to be at least two mechanisms through which silencing of both transgenes and endogenous loci could occur. As defined by these mechanisms, in one class of plants, transgene-induced silencing was accompanied by heavy methylation of silenced loci, presumably leading to a blockade at the transcriptional level (transcriptional gene silencing or TGS; see BOX 1)^{11,12}. In these cases, reversal of methylation by treatment with S-AZADENOXYCYTIDINE relieved repression. A genetic screen in *Arabidopsis thaliana* uncovered two genes, decreased DNA methylation 1 (*DDM1*) and *HOG1*, that are required for maintenance of TGS. Mutation of either of these genes not only relieved repression but also resulted in a loss of methylation at targeted loci¹³. In some cases, however, methylation and silencing could be uncoupled. Disruption of the maintenance of methylation (*MOM*) gene resulted in activation of methylated genes, without affecting methylation. Interestingly, this gene is homologous to the *SWI2* family components of ATP-dependent chromatin-remodelling complexes. This might indicate that a primary enforcer of silencing is altered chromatin structure, which functions with methylation to achieve suppression¹⁴.

In a second class of plant, PTGS occurred. This was clearly shown by NUCLEAR RUN-ON ASSAYS, which indicated that transcript was being made but that it failed to accumulate in the cytoplasm¹⁴. Furthermore, silencing was a systemic phenomenon. Silencing of the endogenous gene could spread from the source plant by grafting tissue onto a host plant that did not contain the transgene¹⁵. A clue to the basis of this phenomenon came

Box 1 | Variations in homology-dependent gene silencing

Homology-dependent gene silencing has been recorded in various organisms, ranging from plants to animals to fungi. Because of the different experimental systems used to study this phenomenon, several terms have emerged, often describing potentially similar processes. These terms are summarized below. Silencing is generally defined by the basis of two properties: the inducing agent and the mechanism of silencing.

Post-transcriptional gene silencing (PTGS)

This is a general term that applies to RNA interference (RNA) in animals, and to some types of virally- and transgene-induced silencing in plants. The transcription of the gene is unaffected; however, gene expression is lost because mRNA molecules become unstable.

Transcriptional gene silencing (TGS)

This is generally observed in plants but has also been seen in animals. Gene expression is reduced by a blockade at the transcriptional level. Evidence indicates that transcriptional repression might be caused by chromatin modification or DNA methylation.

Transgene-induced silencing

Silencing is caused by the presence of transgenes in the genome. Repression is usually related to copy number. Tandemly arrayed transgenes are more effective inducers of silencing than dispersed transgenes, with inverted repeats being the most effective. Silencing can occur transcriptionally or post-transcriptionally.

Virus-induced silencing

Silencing is induced by the presence of viral genomic RNA. Only replication-competent viruses cause silencing, indicating that double-stranded (ds)RNA molecules might be the inducing agents.

Cosuppression

Silencing of an endogenous gene due to the presence of a homologous transgene or virus. Cosuppression can occur at the transcriptional or post-transcriptional level (PTGS or TGS).

RNAi

This type of PTGS is induced directly by dsRNA. It was first defined in *Caenorhabditis elegans* and seems to be mechanistically related, if not identical, to PTGS in plants.

Quelling

This term is specific for transgene-induced PTGS in *Neurospora crassa*.

The insert shows *C. elegans* embryos produced by worms that had been fed BIR-1 or dsRNA. BIR-1 deficiency (right photo) produces a profound cytolysis deficiency as compared with controls (left photo)¹⁵. (Images kindly provided by S. Milstein and M. O. Hengartner.)

from observations made by Baulcombe and colleagues, who were using potato virus X as a vector for generating transgenic plants. It was believed that extremely high levels of transgene expression could be obtained through amplification of the viral RNA by its own RNA-dependent RNA polymerase. Unexpectedly, plants infected with virus vector (containing a reporter) did not yield highly expressing lines. Instead, plants had moderate levels of reporter gene expression, combined with viral resistance. This response, which resembled PTGS, was also effective against genes that contained sequence homology to the viral RNA. As with many RNA viruses, potato virus X generates dsRNA during its replication cycle. Replication defective viruses did not generate this response, indicating the viral RNA was the trigger¹⁶. With hindsight, this was a strong indication that PTGS in plants is similar to RNAi in worms; however, the observations of Baulcombe's group pre-dated the seminal insights of Mello and Fire in *C. elegans*.

It should be noted that suppression by transcription-

al and post-transcriptional mechanisms might not be mutually exclusive. Ultimately, stable and heritable silencing in plants might result from a cross-talk between processes that affect the genomic copy of the gene that is to be silenced and processes that affect the corresponding mRNA¹⁴.

Transgene cosuppression is not limited to plants but has also been shown in fungi, *Drosophila*¹⁷, *C. elegans*¹⁸ and rodent fibroblasts¹⁹. Transgene silencing in the ascomycete fungus, *Neurospora crassa*, had been termed 'quelling' and occurs at the post-transcriptional level (BOX 1). Furthermore, silencing that has been triggered in a transgenic nucleus can spread to a wild-type one in a HETEROKARYON²⁰. In *Drosophila*, single copies of a *white-adt* (for alcohol dehydrogenase) fusion transgene can induce silencing of the endogenous *adt* gene²¹. This effect was more pronounced in flies that were homozygous for the transgene than those that were hemizygous, possibly owing to interaction between the two alleles. This led the authors to suggest that silencing was most probably occurring at the transcriptional level. In a different study, transgenes that were present as an inverted repeat were more effectively silenced than those arranged in direct repeats²¹. This DNA structure, when transcribed, yields RNA in a STEM-LOOP CONFORMATION. As with virally induced gene silencing, it is probable that, at least in the latter case, silencing is triggered by a dsRNA that induces PTGS through the RNAi pathway.

Definitive links between transgene cosuppression and RNAi have come from genetic analysis. Searches for mutant organisms that are resistant to either RNAi or transgene cosuppression (specifically, to quelling) have revealed a common set of molecules. Furthermore, some mutations that prevent RNAi in *C. elegans* also relieve transgene cosuppression¹¹.

It is unclear whether cosuppression that occurs at the transcriptional level or that is provoked by unlinked and single-copy transgenes is related to RNAi. Further investigation is required to reveal the details of these regulatory processes and to illuminate the role of the RNAi pathway in these types of silencing event.

Guide RNAs

As clearly articulated in the original models for RNAi that were proposed by Fire and Mello¹, the most logical way to recognize a target mRNA would be through complementary base pairing. So investigators searched for nucleic acids that were coincident with silencing and that were homologous to the silenced gene. These proved elusive, not so much because of a physically exotic nature but because of their size. The key insight came from Baulcombe and Hamilton, who identified a small RNA species of ~25 nucleotides that was present in plants undergoing either cosuppression or virus-induced gene silencing, but was absent from plants that were not undergoing silencing²². Because the RNAs that triggered silencing were large (>500 nucleotides), the small RNAs could have been participants in the silencing process, products of it, or both. Several lines of evidence indicated a possible integral role. First was the presence of small RNAs that were homologous to both

HETEROKARYON
A cell that contains two nuclei in a common cytoplasm.

STEM-LOOP STRUCTURE
A lollipop-shaped structure that is formed when a single-stranded nucleic acid molecule loops back on itself to form a complementary double helix (stem) topped by a loop.

the sense and antisense strands of the silenced gene, making it unlikely that these were degradation products of the targeted mRNA. Second was the fact that production of the small RNAs did not require the presence of the homologous endogenous gene. In fact, RNA viruses that replicate in the cytoplasm could provoke the production of small RNAs homologous to a gene fragment of their RNA genome. As stated above, silencing by PTGS/RNAi at one site in a plant or worm can spread throughout the organism. Small RNAs could also be found at sites distant from those at which silencing had been induced, and the presence of these species correlated with loss of expression²². So, in plants, the presence of these small RNAs correlated with the silencing process, irrespective of whether silencing had been triggered by cytoplasmic dsRNAs or by genomic transgenes.

The evidence for a general role for small RNAs was provided by the subsequent discovery of these small RNAs in *Drosophila* tissue culture cells in which RNAi had been provoked by introducing large (>500 nucleotide) exogenous dsRNAs²³, in *Drosophila* embryo extracts that were carrying out RNAi *in vitro*²⁴ and in *Drosophila* embryos that had been injected with dsRNA²⁵. The presence of ~25-nucleotide RNAs in flies provided a strong indication that RNAi in animals and PTGS in plants were mechanistically similar, a hypothesis that has been borne out by genetic and biochemical evidence. Recently, small RNAs have been identified in plants undergoing TGS that had been induced by expression of stem loop RNAs²⁶. It should be noted, however, that the data are only correlative. These small RNAs might not actually feed into a transcription-silencing complex, but instead feed into an unobserved PTGS activity. Nevertheless, this finding raises the possibility that a general mechanism applies to PTGS and TGS.

Mechanistic basis of RNAi and PTGS

One of the great mysteries surrounding RNAi is how a cell can respond to virtually any incoming dsRNA by efficiently and specifically silencing genes that are homologous to it. Since the seminal discovery of small

RNA species, progress in both genetic and biochemical dissection of the silencing process has begun to produce a basic understanding of the interference mechanism.

Genetic approaches. Shortly after RNAi was discovered in *C. elegans*, geneticists began to search for mutants that were defective in this response. In parallel, genetic screens were being done in *Neurospora* and *Arabidopsis* and, more recently, in *Chlamydomonas reinhardtii*²⁷, to identify genes that are required for transgene- and virally induced PTGS. Loci are accumulating from all of these systems and are summarized in TABLE 1 as a guide to the discussion below.

Mutants have been identified that affect the formation of silencing activity, the effector step of silencing and the persistence of silencing. These mutants indicate that formation of the silencer and the actual silencing events might be distinct and separable processes. Although none of these mutants has been assigned a definitive mechanistic role in the silencing process, their study has been informative. In particular, mutant plants and animals have additional phenotypes that hint at important biological functions for RNAi and related silencing phenomena.

Two mutants have been shown to affect the formation of silencing activity. These have been placed at the initiation step because heterozygous worms (containing wild-type activity) that have been exposed to dsRNA can transmit RNAi to homozygous mutant offspring, even though homozygous animals can mount no silencing activity in response to an introduced double strand. These are the *C. elegans* RNAi-deficient mutants *rde-1* and *rde-1*. The *rde-1* gene was identified in a screen for first-generation RNAi mutants (rather than mutants in inherited RNAi). The RDE-1 protein is homologous to the quelling-defective (*gde-2*) gene in *Neurospora*²⁸ and to *ARGONAUTE (AGO-1)* in *Arabidopsis*²⁹. Whereas *rde-1* and *gde-2* have no apparent phenotypes other than defects in RNAi or quelling, the *Arabidopsis AGO-1* mutant, which has recently been shown to be defective in PTGS³⁰, has several developmental abnormalities. *AGO-1* mutants have altered leaf differentiation, a reduced number of secondary meristems and are sterile because of defects in flower development³⁰. Mutations in other members of the *Arabidopsis AGO* gene family cause similar developmental effects³⁰, and stem cell phenotypes are also a characteristic feature of *AGO* family mutants in *Drosophila*³¹. The *rde-1* family is evolutionarily conserved, with several genes in all organisms that have been examined, with the exception of *Schizosaccharomyces pombe*. In mammals, a member of the RDE-1 family has been identified as a translation-initiation factor³², and a member of this protein family has been localized to the endoplasmic reticulum in rat cells³³. It is not known whether this protein is involved in RNAi in mammals or whether its function has diverged.

Two mutants have also been identified in the second, effector step of silencing activity; these have been defined as such because a heterozygous worm injected

TABLE 1 | Mutants that resist interference

	<i>Caenorhabditis elegans</i>	<i>Neurospora crassa</i>	<i>Arabidopsis thaliana</i>	Putative function of protein	Involvement of protein in RNAi
<i>rde-1</i>	<i>qde-2</i>	AGO-1	Unknown	Initiation of activity	
<i>rde-2</i>			Not cloned	Effector	
<i>rde-3</i>			Not cloned		
<i>rde-4</i>			Not cloned	Initiation of activity	
<i>mut-2</i>			Not cloned		
<i>mut-7</i>			RNaseD	Effector	
<i>ego-1</i>	<i>qde-1</i>	SGS-2/SDE-1	RdRP		
	<i>qde-3</i>		Werner Bloom helicase		
		SGS-1	Unknown		
		SGS-3	Unknown		

(AGO, argonaute; ego, enhancers of gfp-1; mut, mutator; rde, RNAi-deficient; RdRP, RNA-dependent RNA polymerase; SGS, suppressor of gene silencing; qde, quelling-deficient.)

Box 2 | Biological function of double-stranded RNA-induced silencing

The ability of double-stranded (ds) RNA to provoke gene silencing is a response that has been conserved throughout evolution, indicating it might be important biologically. An often proposed function is as a generalized defence mechanism against unwanted nucleic acids, either in the form of viruses or in the form of parasitic DNA sequences in the genome.

There is a good deal of genetic support for the importance of RNAi and PTGS in genome defence. Many *Caenorhabditis elegans* mutants deficient in RNAi also show transposon mobilization. Similar connections can be drawn in *Drosophila melanogaster* and *Chlamydomonas reinhardtii*^{17,65,67}. Derepression of transposons has obvious implications for genome stability. For instance, mobilized transposons might act as insertional mutagens. Many genomes contain highly repetitive sequences, including transposons, which normally reside in heterochromatin. Derepression of transposons could also disrupt the heterochromatic state and provide homologous sequences for recombination (for example, through double-strand-break repair) between non-homologous regions of chromosomes. In this way, transposon activation could result in large scale destabilization of the genome.

A role for PTGS in transposon control has not yet been observed in plants, but PTGS is clearly involved in the response to RNA viruses. A substantial number of plant viruses have evolved proteins that counter silencing^{29,30}. Many of these viral proteins had been previously identified as pathogenicity determinants, which points to the key role that PTGS has in viral defence. These viral opponents of PTGS are diverse and are likely to interfere at multiple steps in silencing.

The key question that remains unanswered is whether RNAi and related phenomena have a role in regulating the expression of cellular genes. Although there is at present no direct support for this idea, there are indications from the phenotype of RNAi-resistant mutants. In particular, mutation of the *AGO-1* gene in *Arabidopsis thaliana* impairs silencing but also causes numerous developmental abnormalities^{29,30}. So either the silencing process must be important during development or the *AGO-1* protein must have multiple biological functions.

With dsRNA fails to transmit RNAi to homozygous mutant offspring³⁴. Worms with mutations in the RNAi deficient-2 (*rde-2*) and mutator-7 (*mut-7*) genes are less deficient in RNAi than the initiator-step mutants, suggesting that there might be several pathways for silencing. In addition to defects in RNAi, the effector mutants have increased levels of transposon activity and defects in transgene silencing³⁵. These other phenotypes imply that part of the RNAi machinery is involved in silencing transposons and in cosuppression. However, the lack of transposon mobilization in the RNAi-initiation mutants indicates that transposon silencing and RNAi might not occur by completely identical mechanisms. The *rde-2* gene product has not yet been identified. The *mut-7* gene product has homology to both RNase D and to the exonuclease domain of the gene that is disrupted in human Werner syndrome; however, a functional helicase motif that is associated with the Werner protein is apparently lacking³⁶. The Werner syndrome helicase has both DNase and RNase activities^{37–40}, and the MUT-7 protein contains all of the key catalytic residues for nuclease activity. MUT-7 is therefore a reasonable candidate for the catalytic engine of the machinery that destroys mRNAs that have been targeted by RNAi.

Other mutants that are defective in RNAi also show transposon mobilization. These include *rde-3* (REF.35) and *mut-2* (REFS 35,36,41). In addition, these mutations can cause sterility and produce a high incidence of male

offspring (*him* phenotype) that results from spontaneous loss of an X chromosome during meiosis to produce the XO male. The connection between these chromosome-segregation defects and RNAi is not known.

This strong genetic relationship between RNAi and transposon silencing supports the idea that RNAi is involved in genome defence and maintenance of genome stability (BOX 2), and raises questions concerning the route by which transposons and other multicopy genetic elements provoke an RNAi response.

A group of mutants known for their role in *C. elegans* NONSENSE-MEDIATED DECAY have been shown to affect the persistence of interference⁴². These mutants are termed suppressors of morphological effects on genitalia 2,5 and 6 (*sng-2*, *sng-5*, and *sng-6*). The *sng-5* and *sng-6* genes are not cloned, but *sng-2* has homology to a yeast RNA helicase⁴³. No *sng-2* homologues have been discovered in *Neurospora*. However, helicases have been linked to silencing through the *Neurospora* quelling mutant *gde-3* (REF.44). This helicase, like *mut-7*, is related to the human Werner syndrome and Bloom syndrome proteins.

The incredible potency and systemic effects of both RNAi and PTGS have caused many observers to propose a role for RNA-dependent RNA polymerases (RdRPs) in the triggering and amplification of the silencing effect. Whereas RNAi in *C. elegans* is clearly initiated by dsRNA, PTGS in plants can be initiated just by very high levels of expression of a sense-oriented transcript from a transgene. This has led to the proposal that RdRPs recognize 'aberrant' RNAs and convert them to a double-stranded form. One plausible mechanism is that the accumulation of prematurely terminated transcripts provides a source of free 3' hydroxyl termini that are not masked by polyA-binding proteins. These could function as a primer for the creation of a hairpin that would act as the silencing trigger. Whether by producing dsRNA or by amplifying it, the importance of RdRPs is apparent from the discovery of RdRPs among the silencing mutants of *C. elegans*, *Neurospora* and *Arabidopsis*. The *C. elegans* *ego-1* gene was originally identified as a mutation that causes defective germline development⁴⁵. The *ego-1* mutant was subsequently shown to be defective in responses to dsRNAs that are homologous to some genes that are expressed in the germ line⁴⁶ (where *EGO-1* is predominantly expressed). In this mutant, RNAi functions normally for somatic genes. The *Neurospora* *gde-1* mutant, which has a mutation in RdRP, is deficient in quelling but shows no other phenotype⁴⁷. The *Arabidopsis* RdRP *SGS-2/SDE-1* was discovered in two separate screens for PTGS mutants^{48,49}. Interestingly, mutant plants fail to accumulate small RNAs corresponding to the sense transcript of an ectopically expressed gene, but accumulate guide RNAs to an endogenously replicating RNA virus⁴⁸. Correspondingly, *SGS-2/SDE-1* mutant plants can silence a virus that replicates through a dsRNA intermediate but cannot silence a transgene, which might require the RdRP to generate the silencing trigger⁴⁸. It has been proposed that the viral RdRP might substitute for the cellular RdRP in the silencing

NONSENSE-MEDIATED DECAY
The process by which the cell destroys mRNAs that are untranslatable owing to the presence of a nonsense codon within the coding region.

process. However, it is unclear whether the viral RdRP is required solely for production of the viral replication intermediate that functions as a silencing trigger or whether the viral RdRP might also function in the silencing process itself. The apparent absence of a RdRP homologue in the *Drosophila* genome has raised questions about the universality of this aspect of the silencing mechanism. However, a much more complete and detailed analysis will be required to definitely rule out the existence of a fly RdRP.

Biochemical approaches. As genetic routes continue to link proteins to the silencing process, the challenge is to understand precisely how each one fits into the silencing mechanism. In this regard, the development of two related *in vitro* systems is now beginning to shed light on the biochemistry of RNAi. The first was developed by Tuschl and colleagues, who reported that extracts from *Drosophila* embryos specifically prevented the synthesis of luciferase from a synthetic mRNA upon addition of a cognate dsRNA³⁰. The interference activity in the extracts could be amplified by pre-incubation, such that they would still function upon repeated dilution; this was reminiscent of the apparent amplification of the interference effect that occurs during silencing *in vivo*. Finally, the authors showed that inhibition of gene expression was most probably due to degradation of mRNAs that correspond to the added dsRNA.

This study was followed shortly by work from our

laboratory, which reported the development of an *in vitro* system derived from *Drosophila* S2 cells (an embryonic cell line)³¹. The principal difference between the two experimental systems is that, in S2 cells, RNAi is initiated in intact cells by transfection with dsRNA, whereas in the embryo lysates, RNAi is initiated *in vitro* by the addition of dsRNAs.

Our work solidified the idea that RNAi was the result of nuclease digestion of the mRNA but also revealed several key features of the silencing process. The nucleas, which was designated RISC (RNA-induced silencing complex) could be extracted from cells in association with ribosomes. Although this result could be dismissed as an experimental artefact, it was consistent with a previous proposal that RNAi acts as a translational surveillance mechanism. However, neither translation nor capped and polyadenylated mRNAs were not required in either *Drosophila* embryo or S2 cell extracts to observe RNAi *in vitro*³¹.

To test the model that destruction of mRNA was directed by the dsRNA or a derivative thereof, we asked whether the nucleic activity had an integral nucleic acid component. Through partial purification, we found that a ~22-nucleotide RNA that was homologous to the substrate that fractionated together with the RISC and was required for its activity. This led to a model in which the small RNAs that were originally observed in plants functioned as a specificity subunit to direct a multicomponent nuclease towards destruction of homologous mRNAs. Therefore, we coined the name "guide RNAs" for these small RNAs.

As formation of the nuclease had occurred inside transfected cells, it was difficult to determine whether the guide RNAs were derived directly from the incoming dsRNA or derived through replication of the dsRNA by RdRPs, which have been linked to RNAi in other systems. A key insight into this problem came again from the *Drosophila* embryo. Zamore and colleagues, in a follow-up to their earlier study³⁰, identified an activity in *Drosophila* embryo lysates that could cleave long dsRNAs into discrete ~21–23-nucleotide fragments³². This implied that guide RNAs were derived from the dsRNA itself. Erickson and colleagues supported this view by showing that injection of labelled dsRNA into *Drosophila* embryos led to production of labelled guide RNAs³³. Although this did not rule out the possibility that guide RNAs are supplemented by RNAs made by an RdRP, further work indicated that an RdRP activity might not be necessary. Using slightly mismatched dsRNAs, it was shown that the antisense strand is more critical for silencing³⁴. This would make sense if the antisense strand leads directly to guide RNAs, which results in targeting of the sense transcript by hybridization. If replication was taking place, either strand could lead ultimately to homologous antisense guide RNAs.

In support of the model that these small RNAs guide mRNA destruction, Zamore and colleagues showed that the dsRNA determined the boundaries of cleavage of the mRNA and that the mRNA was also cut at 21–23-nucleotide intervals³². The latter observation implies

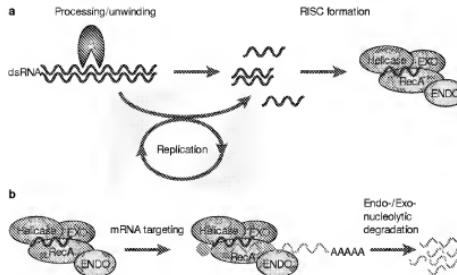
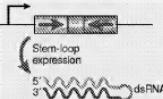
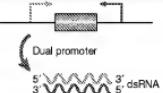


Figure 1 | How does RNAi work? Genetic and biochemical data indicate a possible two-step mechanism for RNA interference (RNAi): an initiation step and an effector step. **a** In the first step, input double-stranded (ds) RNA is processed into 21–23-nucleotide 'guide sequences'. Whether they are single- or double-stranded remains an open question. An RNA amplification step (shaded box) has been suggested on the basis of the unusual properties of the interference phenomenon in whole animals, but this has not been reproduced definitively *in vitro*. **b** The guide RNAs are incorporated into a nucleic acid complex, called the RNA-induced silencing complex (RISC), which acts in the second effector step to destroy mRNAs that are recognized by the guide RNAs through base-pairing interactions. We also suggest the incorporation of an active mechanism to search for homologous mRNAs. (Endo, endonucleolytic nuclease; exo, exonucleolytic nuclease; recA, homology-searching activity related to *E. coli* recA.)

Animated online

Box 3 | Delivering double-stranded RNA

Method	Organism	Pros	Cons
	<i>C. elegans</i> <i>Drosophila</i> Trypanosomes	• Fast • Effective • Works in many systems	• Non-inducible • Most effective in embryonic systems
	<i>C. elegans</i> ⁷⁷ <i>Drosophila</i> ⁵⁵ Trypanosomes ⁷⁸ Plants ⁷⁹	• Stable • Inducible • Tissue specific	• Time consuming to generate • Cloning can be problematic
	Trypanosomes ^{78,80}	• Stable • Inducible • Tissue specific	• Time consuming to generate • Promoters can silence each other
	Plants	• Most common technique in plants	• Limited to plants at present

The original protocol for initiating RNA interference (RNAi) in *Ceaeorhabditis elegans* involved the direct introduction of double-stranded (ds)RNA by injection. By contrast, PTCS could be most easily induced in plants by infection with potato virus X. Although these two methods are still highly effective in their respective organisms, several other approaches have been developed for these, and for other organisms (including *Drosophila melanogaster*, see figure above). These permit initiation of RNAi at specific developmental stages, or in selected tissues. One way of achieving this involves expressing an RNA molecule that folds into a stem-loop structure, which mimics a double-stranded RNA. Alternatively, both (sense and antisense) strands of the dsRNA can be transcribed using separate promoters. With inducible, or tissue-specific promoters, the RNAi response can be tailored to the experimental requirements. Although both approaches have met with success, the stem-loop strategy is more widespread.

REKA (oxA)
A multifunctional protein in *E. coli* that is involved in DNA recombination and postreplicative DNA repair. This protein also has an energy-dependent homology-searching activity.

that, at some step in the pathway (either during generation of the 22-nucleotide fragment or mRNA cleavage by RISC), a nuclease might act processively on the substrate. These investigators also showed that RNAi was ATP-dependent. Although it was not completely clear which step of the process required ATP, the authors did show that the production of guide RNAs was greatly attenuated in the absence of ATP.

These initial forays into the biochemistry of RNAi have yet to link genetically identified players to specific roles in the silencing process. However, purification of both the RISC and of the nuclease that produces guide RNAs is continuing. It is likely that these efforts will begin to forge links between the biochemical

mechanisms determined *in vitro* and the genetic analyses of diverse systems. An additional benefit could come from the development of *in vitro* assays from systems such as *C. elegans* or *Arabidopsis*, in which genetic mutants already exist, or by the identification of RNAi-resistant mutants in *Drosophila*.

Current model

The biochemical analyses described above have given us an understanding of the interference process that is sufficient to build at least a superficial model (FIG. 1). The trigger for silencing by RNAi (and perhaps also in some cases of transgene-induced cosuppression) is clearly dsRNA. This is recognized by a nuclease that cleaves the dsRNA into ~21–23-nucleotide fragments. The prototypical nuclease that is specific for dsRNA belongs to the RNaseIII family of enzymes, which have been proposed to be components of RNAi^{51,52}. The first biochemical measurement of such an enzyme was reported by Zamore and colleagues⁵³, and a candidate protein has been identified by our lab⁵⁴. Guide RNAs are assembled with one or more protein components to form the RISC. The RISC enzyme uses the instruction provided by the guide RNAs to identify mRNA substrates for degradation.

Although the model accounts for the basic observations that have been made *in vitro*, it fails to account for many of the unusual properties of these silencing phenomena *in vivo*. For example, it is unclear how the RISC enzyme can identify its substrates so efficiently. The limited efficacy of antisense RNAs in many organisms suggests that base-pairing between complementary RNAs *in vivo* is not a common or favoured situation. So it seems likely that the RISC enzyme contains some mechanism to search the cellular transcriptome for its specific substrates, similar to the ability of *ricea* family proteins to search genomic DNA for homologous sequences⁵⁴. Furthermore, the model makes no attempt to account for the systemic nature and heritability of silencing. However, we are optimistic that, as the biochemistry and genetics of the interference process evolve, we might be able to fit these unusual properties into a more detailed description of the silencing process.

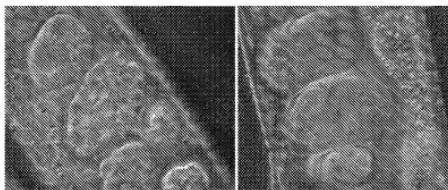
RNAi today and in the future

The use of RNAi as a method to alter gene expression has been attempted in a wide variety of organisms, using different methods (BOX 3) and with varying degrees of success (TABLE 2). In *C. elegans*, *Drosophila* and plants, RNAi seems to be an effective, specific and therefore valuable tool for reverse genetics. A second class of organism, including zebrafish, *Xenopus* and mouse, has been shown to have some capacity for RNAi; however, there seem to be significant limitations.

In almost all cases, treatment of worms with dsRNA gives a phenocopy of the genetic null mutant (BOX 4). Typically, adult hermaphrodites are soaked with dsRNA or are fed *E. coli* cells that express the dsRNA. Specificity is usually high, although closely related sequences can

Table 2 | RNA interference as a tool in various organisms

RNAi as a tool today	Works, but some limitations	RNAi in the future
<i>C. elegans</i>	Zebrafish	Mammalian cultured cells
<i>Drosophila</i>	<i>Xenopus</i>	Mouse
Plants	Mouse embryo	Human?
<i>Planaria</i>		
Trypanosome		

Box 4 | What does RNA interference do for me?

The discovery of RNA interference (RNAi) has revolutionized reverse-genetic approaches in diverse systems. RNAi has become a standard procedure in *C. elegans* (see above figure) and is being harnessed in large-scale genomics efforts in plants. It has also allowed the functional analysis of genes in *Drosophila melanogaster* and cultured *Drosophila* cells, and in more exotic organisms such as planaria and trypanosomes^{60,71}. RNAi has been used successfully in *Drosophila*, particularly for the study of embryonic phenotypes. However, recent demonstrations that enforced production of hairpin RNAs in adults can silence gene expression promise to expand the utility of this approach⁷².

Several recent papers have suggested that RNAi might also prove useful in vertebrate systems. The first indication came from zebrafish, in which silencing was observed after injection of double-stranded (ds)RNA into embryos^{73,74}. The effect seemed less potent than that seen in *C. elegans*, with only 20–30% of embryos being affected.

Subsequently, concerns about cross-interference have slowed the acceptance of this technology⁷⁵, so further work is needed before drawing a conclusion regarding the utility of RNAi in zebrafish.

The report that dsRNA can specifically interfere with gene expression in mammals was more startling. Injection of dsRNA into early stage mouse embryos specifically antagonized both endogenous and exogenous genes^{63,64}. The effects, however, were transient, lasting roughly until the time of implantation. This raises the possibility that increased knowledge of the mechanisms of RNAi will provide the means to prolong the effect and make dsRNA-induced gene silencing a generally useful tool for probing gene function in mammals. The insert shows *C. elegans* embryos produced by worms that had been fed BIR-1 or dsRNA. BIR-1 deficiency (right photo) produces a profound cytokinesis deficiency as compared with controls (left photo)⁷⁶. (Images kindly provided by S. Milstein and M. O. Hengartner.)

cross-interfere⁸. Alternatively, worms can be engineered that express a stem-loop RNA from a stable episome or from genomic integrants⁸⁵. This allows the generation of stable phenotypically null strains. Although RNAi has been most widely harnessed for reverse genetics, whole-genome approaches are on the horizon. Recent reports have described the use of RNAi to systematically and individually ablate each gene on entire *C. elegans* chromosomes^{65,67}.

RNAi technology is proving beneficial for the study of *Drosophila* embryology. Injection of early stage embryos leads to null phenotypes at later stages. But this approach is restricted to a relatively narrow developmental window. A more general approach to achieve gene silencing in embryos or in adult flies is through the expression of stem-loop RNAs; however, this requires the time consuming operation of generating transgenic animals⁸⁶. Finally, RNAi is being harnessed for reverse genetics in cultured *Drosophila* cells. This process is almost trivial because silencing can be achieved simply

by adding the dsRNA to the culture medium⁸⁹.

Among the reasons that both *C. elegans* and *Drosophila* have benefited so markedly from RNAi is the difficulty (in the case of *C. elegans*, the impossibility) of generating targeted null mutations analogous to mouse knockouts⁶⁰. This is also the case in planaria and trypanosomes, two other organisms in which RNAi technology can be applied^{61,62}.

The related phenomenon of virus-induced gene silencing is a potentially powerful approach for plant genetics. Introducing the inducing agent can be done in many ways. One option is to engineer the plant vector *Agrobacterium tumefaciens* to deliver a hairpin expression construct, and another is to use plant viruses such as potato virus X. In the latter case, dsRNA that is generated during viral replication in the plant silences the endogenous genes that are homologous to any sequences carried within the virus. These technologies will encourage loss-of-function RNAi screens to be done in plants, similar to those being done in *C. elegans*⁸⁷.

Many investigators are eager to see RNAi technologies applied as genetic tools in vertebrate organisms (TABLE 2). Some hope for this possibility is provided by the discovery that dsRNA can induce sequence-specific silencing in early mouse embryos^{60,61}. Unfortunately, the use of this approach is, at present, limited to a narrow developmental window, and effects provoked in early embryos do not persist after implantation⁶¹. It has recently been reported that dsRNAs can silence the expression of exogenous genes in Chinese hamster ovary cells⁶⁰. Although it has yet to be definitely shown that the mechanisms by which dsRNAs induce silencing in mammals are similar to those that seem to be conserved in plants, flies, worms and fungi, it seems probable that the interference mechanism is universal. So a deeper understanding of RNAi in various experimental systems might lead to insights into how to harness this potentially latent process in mammalian cell culture and in mammalian animals.

Conclusions

Since the independent discovery of seemingly unrelated silencing phenomena in plants, fungi and animals, we have come to realize that dsRNA can activate an evolutionarily conserved mechanism of gene regulation. Over the past few years, we have come to understand the basic mechanism through which silencing occurs. However, only through a combination of biochemical and genetic approaches in several experimental models can we hope to elucidate the mechanistic basis of dsRNA-induced silencing and to appreciate its biological importance.

Links

DATABASE LINKS par-1/DDM1/rde-4/rde-11/rde-21AGO-1/rde-21mut-71Werner syndrome 1mut-21smg-21smg-51smg-61qde-31Bloom syndrome 1ego-11SGS-21SDE-1

1. Fire, A. et al. Potent and specific genetic interference by double-stranded RNA in *C. elegans* elegans. *Nature* **391**, 806–811 (1998).
- This is the first paper to describe the phenomenon of RNA interference (RNAi).
2. Tabara, H., Grishok, A. & Schedl, P. Scanning in the genome sequence. *Science* **282**, 430–431 (1998).
3. Guo, S. & Kemphues, K. J. par-1, a gene required for establishment polarity in *C. elegans* embryos, encodes a putative protein kinase that is asymmetrically distributed. *Cel* **81**, 611–620 (1995).
4. Williams, B. R. PKR, a sentinel kinase for cellular stress. *Oncogene* **18**, 611–6120 (1999).
5. Stuurman, N. C., Williams, B. R., Silverman, R. H. & Schieren, R. D. How specific is silencing? *Annu. Rev. Biochem.* **67**, 237–264 (1998).
6. Montgomery, M. K., Xu, S. & Fire, A. RNA as a target of double-stranded RNA-mediated genetic interference in *C. elegans* elegans. *Proc. Natl. Acad. Sci. USA* **95**, 18502–18507 (1998).
7. Bracht, J., M. Dufourcq, P. Sookhdeo, S. & Labouesse, M. RNAi interference can target pre-mRNA consequences for gene expression in a *C. elegans* elegans. *Genetics* **135**, 1245–1256 (1993).
8. Pandey, S., Bhattacharya, T., Bhattacharya, S. & Fire, A. Functional anatomy of a dsRNA trigger: minimal requirement for the two triggers in RNA interference. *Mol. Cell* **8**, 1077–1087 (2000).
9. Oue, R. & Jorgensen, R. A. Homology-based control of gene expression patterns in *C. elegans* nematocysts. *Dev. Cell* **1**, 103–113 (2001).
10. Jorgensen, R. A.,uster P. D., Engels, J., Quee, O. & Napoli, C. A. Chitosan synthase cossuppression phenotypes in *C. elegans* flowers: comparison of sense vs antisense constructs and single-copy vs copy-T-DNA sequences. *Plant Mol. Biol.* **39**, 165–176 (1999).
11. Amodeo, P., Habu, Y., Alzari, K., Scheid, O. M. & Paszkowski, J. Disruption of the plant gene *MOM* releases transcriptional silencing of methylated genes. *Nature* **399**, 236–239 (1999).
12. Ingebretsen, L., Van Houtte, H., Van Montagu, M. & Depicker, A. Posttranscriptional silencing of reporter transgenes in tobacco correlates with DNA methylation. *Proc. Natl. Acad. Sci. USA* **91**, 10502–10506 (1994).
13. Padovan, M., Amodeo, P., Scheid, O. M. & Paszkowski, J. Transiently acquired silencing: heterologous-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced ones. *EMBO J.* **16**, 4738–4745 (1997). *erratum* **17**, 2137 (1998).
14. Amodeo, P. & Paszkowski, J. Consistent generation in transgenic plants of a heterologous silencing potato virus RNA. *EMBO J.* **16**, 3675–3684 (1997).
15. Pal-Bhadra, M., Bhadra, U. & Brunner, J. A. Coexpression in *Drosophila* gene silencing of Alcohol dehydrogenase by *dsRNA* transgenes is Polycomb dependent. *Proc. Natl. Acad. Sci. USA* **96**, 14790–14795 (1999).
16. Denburg, A. F., Zawieja, J., Colicosova, M. P. & Vilimovcova, A. M. Transgene-mediated coexpression in the *C. elegans* germ line. *Gene Dev.* **14**, 1578–1583 (2000).
17. Baumlein, M. B. & Zaitsev, H. Transcriptional and posttranscriptional silencing of related alpha 1(I) collagen by a heterologous-specific posttranscriptionally silenced transgene. *Mol. Cell. Biol.* **19**, 274–283 (1999).
18. Cogoni, C. et al. Transgene silencing of the *al-1* gene in vegetative cells of *Neurospora* is mediated by a cytosolic endoribonuclease. I. Characterization of the integral 3'-5' exonuclease. *J. Biol. Chem.* **273**, 3153–3163 (1998).
19. Dorer, D. R. & Henikoff, S. Expansions of repetitive elements cause heritable epigenetic changes and gene silencing. *Cell* **95**, 903–909 (1998).
20. Hamilton, A. J. & Baulcombe, D. C. A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* **286**, 950–952 (1999).
- A potential mediator of post-transcriptional gene silencing (PTGS) has been found for the first time. This small ~25-nucleotide RNA has become a hallmark of silencing processes that are mediated by double-stranded (ds) RNA molecules.
21. Hammond, S. M., Bernstein, E., Beach, D. & Horwich, G. J. An RNA-directed nucleic acid mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature* **404**, 293–296 (2000).
- The authors identify a dsRNA-inducible nuclease activity that targets mRNAs for degradation. They also show that this enzyme is associated with -22 -nucleotide double-stranded RNAs (guide RNAs) that might determine target specificity.
22. Zamore, P. D., Tuschl, T., Sharp, P. A. & Bartel, D. P. RNA double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101**, 253–263 (2000).
- By developing their earlier work on an *in vitro* RNAi system, these authors show that ~ 22 -nucleotide guide RNAs can produce directly from longer dsRNAs *in vitro* and that target mRNAs are cleaved in regions corresponding to the 21-nucleotide intervals.
23. Yang, D., Li, H. & Erickson, J. W. Evidence that posttranscriptional gene silencing may mediate sequence specific mRNA degradation during RNAi in *Drosophila* embryos. *Curr. Biol.* **10**, 1191–1200 (2000).
24. Mette, M. F., Aufsat, W., van der Winden, J., Matzke, A. M. & Matzke, J. A. Posttranscriptional silencing and paramutation bypassed by double-stranded RNA. *EMBO J.* **19**, 5194–5201 (2000).
25. Wu-Chien, D., Jeong, B., Zhang, C. & Cenati, H. Transgene and transposition silencing in chlamydomonas reinhardtii are mediated by a DEAF-Box RNA helicase. *Science* **290**, 1159–1163 (2000).
26. Cottrell, G., Aranzini, G., Manica, G. & Cognetti, G. Gene silencing in worms and in plants. *Nature* **404**, 245 (2000).
27. Nagagi, M., Bourque, S., Moret, J. B., Barbin, C. & Vassilatis, H. ADD-2, QDE-2, and RDE-1 are related proteins required for post-transcriptional gene silencing in plants, queuing in fungi, and RNA interference in animals. *Proc. Natl. Acad. Sci. USA* **97**, 11650–11654 (2000).
28. Bohmert, K. et al. QDE-1 defines a novel locus of *Arabidopsis* controlling leaf development. *EMBO J.* **17**, 1705–1715 (1998).
29. Lin, H. & Spradling, A. C. A novel group of *pumilio* mutations affects the symmetric division of germline stem cells in the *Drosophila* ovary. *Development* **124**, 2445–2455 (1997).
30. Zeng, C., Zhou, Z., Wu, S. & Osterman, J. C. Molecular cloning and characterization of a rabbit eIF2C protein. *Gene* **211**, 187–194 (1995).
31. Cukajuk, E. C. et al. GEP-P95, a membrane-associated protein that belongs to a family of proteins involved in stem cell differentiation. *Mol. Biol. Cell* **10**, 3357–3372 (1999).
32. Gherardi, A., Tabara, H. & Melo, C. C. Genetic requirements for inheritance of RNAi in *C. elegans*. *Science* **287**, 2494–2497 (2000).
33. Tabara, H. et al. The rde-1 gene, RNA interference, and transposon silencing in *C. elegans*. *Nature* **399**, 123–125 (1999).
- With reference 26, this is one of the first reports of the genetic analysis of RNAi in *C. elegans*. This paper provides evidence that a homolog of ARIONAUTA family of proteins is involved in the silencing process.
34. Ketting, R. F., Haverkamp, T. H., van Luenen, H. G. & Plasterk, R. H. Mu-7 dC₃ elements, required for transposon silencing, RNA interference, and transposon silencing in *C. elegans*. *Nature* **399**, 123–141 (1999).
- Establishes a link between RNAi and transposon silencing and identifies a putative nuclease as an effector of silencing.
35. Suzuki, K., Yamada, K., Moto, M., Furukawa, Y. Werner syndrome helicase contains a 5'-3' exonuclease activity that digests DNA and RNA strands in DNA/RNA and DNA/RNA duplexes dependent on unwinding. *Nucleic Acid Res.* **27**, 2261–2268 (1999).
36. Kamath, R. S., Shen, J. C., Loeb, L. A. & Fly, M. Werner syndrome helicase and RNaseH2. Characterization of the integral 3'-5' exonuclease. *J. Biol. Chem.* **273**, 34145–34150 (1998).
37. Shen, J. C. et al. Werner syndrome protein 1 DNA helicase and DNA exonuclease reside on the same polypeptide chain. *Chem.* **27**, 3411–3414 (1998).
38. Huang, S. et al. The protein-coding gene Werner protein, WRN, is a 3'-5' exonuclease. *Nature Genet.* **20**, 114–116 (1996).
39. Collins, A. & Anderson, K. B. A 765 family of transposons in *C. elegans*. *Genetics* **137**, 771–781 (1994).
40. Domene, M. E. et al. A link between RNA interference and nonsense-mediated decay in *C. elegans* elegans. *Science* **289**, 1928–1931 (2000).
41. Page, M. P., Carr, B., Anders, K. R., Grimes, A. & Anderson, S. SMO-2 is a phosphorylated protein required for mRNA surveillance in *C. elegans* elegans and related to Upf1p of yeast. *Mol. Cell. Biol.* **19**, 5943–5951 (1999).
42. Cogoni, C. & Macino, G. Posttranscriptional gene silencing in *Neurospora* by a *dsRNA* helicase. *Science* **284**, 2242–2244 (1999).
43. Oiso, L. et al. Enhancers of *glp-1*, a gene required for cell signaling in *C. elegans* elegans, define a set of genes required for gamete development. *Genetics* **141**, 561–569 (1995).
44. Smardon, A. et al. EGO-1 is related to RNA-directed RNA polymerase and functions in germ-line development and RNA interference in *C. elegans*. *Curr. Biol.* **10**, 169–178 (2000); erratum in **10**, F293–F294 (2000).
45. Cogoni, C. & Macino, G. Germ-line development in *Neurospora* requires a protein homologous to RNA polymerase. *Nature* **399**, 165–169 (1999).
46. Daemm, T., Hammon, A., Ravid, S., Angel, S. & Baulcombe, D. C. An RNA-dependent RNA polymerase gene in *Arabidopsis* is required for posttranscriptional gene silencing. *Proc. Natl. Acad. Sci. USA* **96**, 1165–1170 (1999).
47. Smardon, A. et al. EGO-1 is related to RNA-directed RNA polymerase and functions in germ-line development and RNA interference in *C. elegans* but not by a dsRNA. *Curr. Biol.* **10**, 543–553 (2000).
48. Mourant, P. et al. *Arabidopsis* SG3 and SG5 genes are required for posttranscriptional gene silencing and natural virus resistance. *Curr. Opin. Plant Biol.* **10**, 533–542 (2000).
49. Turner, J. P. & Vaucheret, H. The RNAi pathway in *Arabidopsis*. *Trends Plant Sci.* **5**, 176–182 (2000).
50. Baulcombe, P. Targeted mRNA degradation by double-stranded RNA in *Arabidopsis*. *Genes Dev.* **13**, 3191–3197 (1999).
51. Bass, B. L. Double-stranded RNA as a template for gene silencing. *Cell* **101**, 235–238 (2000).
52. Cenati, H. M. & Cukajuk, E. C. Domains in gene silencing and posttranscriptional genes: the novel PAZ domain and redefinition of the pen domain. *Trends Biochem. Sci.* **25**, 481–482 (2000).
53. Bernstein, E., Caudy, A. C., Hannon, G. J. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**, 363–366 (2001).
54. Cenati, H. M. et al. RNA-induced gene reactivation and recombination-dependent regulation. *Trends Biochem. Sci.* **25**, 156–165 (2000).
55. Tsvetkov, N., Wang, S. L., Dorovkina, M., Ryazanova, A. & Demjanov, M. A. RNAi can induce genetic interference in double-stranded RNA encoded by transgenes. *Nature* **24**, 180–183 (1993).
56. Fraser, A. G. et al. Functional genome analysis of *C. elegans* chromosome X by systematic RNA interference. *Nature* **406**, 325–330 (2000).
57. Cenati, H. M. et al. Systematic analysis of cell division in *C. elegans* using RNAi of genes on chromosome III. *Nature* **406**, 331–336 (2000).
58. Kemens, J. H. & Carthew, R. W. Heritable gene silencing in *Drosophila* using double-stranded RNA. *Proc. Natl. Acad. Sci. USA* **97**, 14691–14695 (2000).
59. Clements, J. C. et al. Use of double-stranded RNA interference in *Drosophila* cell lines to dissect signal transduction pathways. *Proc. Natl. Acad. Sci. USA* **97**, 6499–6503 (2000).
60. Rong, Y. S. & Golz, K. G. Gene targeting by homologous recombination in *Drosophila*. *Science* **288**, 2013–2018 (2000).
61. Ngo, H., Techiishi, C., Guk, K. U. & Shultz, R. M. Identification of domains of maternal mRNA in mouse eggs by RNA interference. *Development* **127**, 4147–4156 (2000).
62. Wanny, F. & Zemlicka-Gozt, M. Specific interference with gene function by double-stranded RNA in early mouse development. *Nature Cell Biol.* **2**, 75–79 (1999).
63. Liu, Y., Xiang, S. M. & Zhou, Y. S. Genetic assay of RNA interference in *Drosophila* and Chinese hamster cultures using tetracycline inducer gene as target. *FEBS Lett.* **479**, 79–82 (2000).
64. Jensen, S., Geissler, M. P. & Heidmann, T. Identification of transposon activity in *Drosophila* containing sense and antisense transgenes. *Genetics* **153**, 1767–1774 (1999).
65. Malesky, S., Bucheton, A. & Bussuel, J. New insights on homology-dependent regulation of factor activity by transposons. *Cell* **75**, 735–745 (1993).
66. Baulcombe, D. C. *Arabidopsis* CRISPR/Cas9. *Genetics* **156**, 1147–1151 (2000).
67. Bignotti, G. et al. Wall-pathway determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. *EMBO J.* **17**, 6739–6746 (1998).
68. Voinnet, O., Prato, Y. M. & Baulcombe, D. C. Suppression of gene silencing: a general strategy used by diverse DNA and RNA viruses of plants. *Proc. Natl. Acad. Sci. USA* **96**,

- 14147–14152 (1999).
- This is one of several papers that establishes a second biological function for RNA by linking dsRNA-induced silencing to virus resistance in plants.
70. Ruiz, F., Voyatzis, L., Klitz, C., Sperling, L. & Madeddu, L. Homology-dependent gene silencing in *Paramecium*. *Mol. Biol. Cell* **9**, 931–943 (1998).
71. Shi, H. et al. Genetic interference in *Trypanosoma brucei* by heritable and inducible double-stranded RNA. *RNA* **6**, 1000–1006 (2000).
72. Kennedy, G. R. & Carthew, R. W. Heritable gene silencing in *Drosophila* using double-stranded RNA. *Nature Biotechnol.* **18**, 895–898 (2000).
73. Li, Y. X., Farrell, M. J., Luu, R., Mohanty, N. & Kirby, M. L. Double-stranded RNA injection produces null phenotypes in zebrafish. *Dev. Biol.* **217**, 394–405 (2000); erratum **220**, 432 (2000).
74. Wargelius, A., Ellingsen, S. & Fjose, A. Double-stranded RNA induces specific developmental defects in zebrafish embryos. *Biochem. Biophys. Res. Commun.* **263**, 156–161 (1999).
75. Oates, A. C., Burke, A. E. & Ho, R. K. Too much interference: induction of double-stranded RNA has nonspecific effects in the zebrafish embryo. *Dev. Biol.* **224**, 20–28 (2000).
76. Frisee, A. G., James, C., Evan, G. I. & Hengartner, M. O. Caenorhabditis elegans inhibitor of apoptosis protein (IAP) homologue BIR-1 plays a conserved role in cytolysis. *Curr. Biol.* **9**, 103–106 (1999).
77. Tanguay, R. M., Wang, S. L., Doevikov, M., Pyatkov, A. & Driscoll, M. Heritable and inducible genetic interference by double-stranded RNA encoded by transgenes. *Nature Genet.* **24**, 180–183 (2000).
78. Shi, H. et al. Genetic interference in *Trypanosoma brucei* by heritable and inducible double-stranded RNA. *RNA* **6**, 1069–1076 (2000).
79. Chuang, C. F. & Meyerowitz, E. M. Specific and heritable genetic interference by double-stranded RNA in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* **97**, 4985–4990 (2000).
80. Wang, Z., Morris, J. C., Drew, M. E. & Englund, P. T. Interference of *Trypanosoma brucei* gene expression by RNA interference using an integratable vector with opposing T7 promoter. *J. Biol. Chem.* **275**, 40174–40179 (2000).

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